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New records of the rare deep-water alga *Sebdenia monnardiana* (Rhodophyta) and the alien *Dictyota cyanoloma* (Phaeophyceae) and the unresolved case of deep-water kelp in the Ionian and Aegean Seas (Greece)

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New records of the rare deep-water alga *Sebdenia monnardiana* (Rhodophyta) and the alien *Dictyota cyanoloma* (Phaeophyceae) and the unresolved case of deep-water kelp in the Ionian and Aegean Seas (Greece)

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Abstract: Parts of the macroalgal flora of the eastern Mediterranean remain incompletely known. This applies in particular to the circalittoral communities. This study, based upon 2 cruises in the Ionian and Aegean Seas, surveyed benthic communities from 40 to 150 m depth by remotely-operated vehicle (ROV) with a special focus on detecting communities of the Mediterranean deep-water kelp *Laminaria rodriguezii*. These were complemented by shallow-water surveys on adjacent coastlines by snorkelling and scuba diving. While no kelp could be detected at any of

the sites surveyed, ROV surveys of northern Euboea Island revealed the first east Mediterranean record of *Sebdenia monnardiana* (Sebdeniales, Rhodophyta). Snorkelling surveys on the coast of southeast Kefalonia yielded the first record of the alien alga *Dictyota cyanoloma* in Greece. This paper reports *rbcL* and SSU sequences for *Sebdenia monnardiana*, and COI for *Dictyota cyanoloma*.

Keywords: alien species; COI; *Laminaria rodriguezii*; *rbcL*; SSU.

Introduction

About 270 taxa of brown algae have been recorded until today in the Mediterranean Sea (Ribera et al. 1992, Cormaci et al. 2012). However, when it comes to the eastern basin, the number is far lower. It is not clear whether this is actually due to a lower algal diversity in this part of the Mediterranean Sea or whether it rather reflects fewer biodiversity studies in the eastern in comparison to the Western basin.

Several misidentifications and unconfirmed records as well as recent findings of new species suggest still significant gaps in the knowledge of seaweed diversity of the eastern Mediterranean basin (Tsiamis and Panayotidis 2016). Indeed, a recent increase in phycological expeditions and overall research effort in the eastern basin has revealed numerous new macroalgal records, in particular for Greece such as *Discosporangium mesarthrocarpum* (Meneghini) Hauck (Tsirika and Haritonidis 2005), *Cystoseira schiffneri* Hamel [as *Cystoseira foeniculacea* f. *schiffneri* (Hamel) Gómez Garreta, Barceló, Ribera et Rull Lluch], and *Cystoseira funkii* Schiffner ex Gerloff et Nizamuddin (Tsiamis et al. 2016), Cyprus (Tsiamis et al. 2014) and Turkey (Taskin and Ozturk 2007, 2008, Taskin 2008, 2013). Recent findings also include numerous new records of alien species (Tsiamis et al. 2010, 2013), with *Caulerpa taxifolia* var. *distichophylla* (Sonder) Verlaque, Huisman et Procaccini occurring over an exceptionally wide depth

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range (Aplikioti et al. 2016). Also, the combined use of algal culturing and molecular techniques (in particular, the Germling Emergence Technique) has unravelled additional new records and hitherto-undescribed taxa from the eastern Mediterranean (Peters et al. 2015).

Populations of the Mediterranean endemic deep-water kelp *Laminaria rodriguezii* Bornet are found in the western Mediterranean and the Adriatic Sea (Guiry and Guiry 2019). The species may exist also in the eastern basin but still remains undetected. Reports without description or illustration from Turkey (Aysel et al. 2008, Taskin and Öztürk 2013) need to be confirmed by new collections. When it comes to the Adriatic Sea, extensive surveys demonstrated a significant decline in the range of the species due to bottom trawling (Žuljević et al. 2016).

Recently, a computer model was developed which is capable of predicting deep-water kelp habitats in warm-temperate and tropical seas (Graham et al. 2007). At the onset of this study, it appeared as an attractive tool to select target areas for ship-based surveys.

The surveys described here were conducted within the framework of two cruises in the Ionian and Aegean Sea (Greece), aiming to elucidate the potential presence of deep-water kelp communities in the eastern Mediterranean Sea using remotely-operated vehicles (ROVs). In addition, dingy- and shore-based snorkelling and scuba-diving surveys of adjacent littoral habitats of interest took place in order to study the macroalgal diversity in more shallow waters. While *Laminaria rodriguezii* was

not detected, we here report new records from Greece of two poorly known taxa, including DNA sequence data: *Dictyota cyanoloma* Tronholm, De Clerck, Gómez-Garreta et Rull Lluch and *Sebdenia monnardiana* (Montagne) Berthold.

Materials and methods

ROV and diving surveys

Macroalgal surveys were conducted from the R/V Philia (Hellenic Centre for Marine Research) from March 10–17 to October 20–25, 2013. Surveys utilised a remotely operated vehicle (ROV, SAAB Seaeye Falcon, rated to 300 m depth; www.seaeye.com) equipped with a video camera and robotic arm capable of collecting sessile organisms. In addition, scuba diving and snorkelling in adjacent infralittoral waters were performed, using the dingy of the R/V Philia as a support platform. Target areas for *Laminaria rodriguezii* had been chosen using maps (Figure 1) based upon the prediction model of deep-water kelp habitats based on water transparency and temperature data, previously used in tropical seas (Graham et al. 2007). The model combined satellite-derived data on water transparency and incident irradiance with locally-derived data on bathymetry and water temperature depth profiles to estimate the extent of substrate with enough light to support

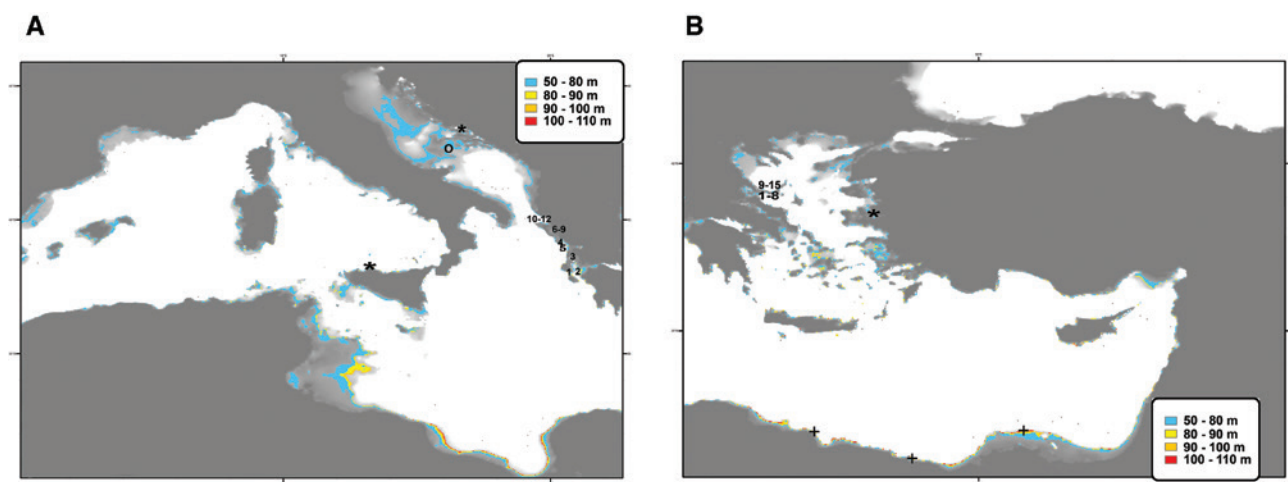


Figure 1: Prediction maps of the potential occurrence of *Laminaria rodriguezii* in the Eastern Mediterranean.

Coloured shading indicates potential deep-water kelp habitat colour-coded by depth range. These grid cells met all model criteria for kelp occurrence. Grid cells are approximately 3.8×3.8 km². Predicted kelp habitat shallower than 50 m is not shown. Black crosses show locations of predicted kelp habitat deeper than 110 m. Grey shading shows ETOPO2v2 bathymetry from 0 m (dark grey shading = land) to the 200 m isobath (lightest grey shading). The numbers correspond to the ROV surveys detailed in Table 1. O denotes the Jabuka area, where *L. rodriguezii* has been documented (Žuljević et al. 2016), while asterisks denote the sites nearest to the ones reported in this study where *Dictyota cyanoloma* was found. (A) Central Mediterranean. (B) Eastern Mediterranean.

kelp productivity that existed below the mixed layer depth. This area would be considered the most likely to support deep *L. rodriguezii* populations, regardless of the quality of the substratum (i.e. soft- vs. hard-bottom).

Surveys in March 2013 spanned the waters from Patras and Kefalonia in the south to Corfu (Kerkyra) in the north, while the surveys in October 2013 extended from Aidipos (Euboia Island) towards Oreoi, along the mainland coast of Pelio and around the Northern Sporades Islands (Figure 1 and Table 1 for all stations of ROV surveys). These were complemented by snorkelling surveys at sites on adjacent coasts, in particular the surroundings of Argostoli and the coast of southeast Kefalonia, aiming to study the overall shallow algal biodiversity.

Collection of materials

Seaweed specimens were collected as entire thalli either by the robotic arm of the Seaeye Falcon™ ROV, by snorkelling or by SCUBA diving. They were subsequently conserved as herbarium specimens on Bristol paper or on microscope slides using acetocarmine as fixative and dye and 50% Karo™ syrup as mounting medium (Müller and Ramírez 1994).

Seaweed tissue samples were conserved in parallel in Silica gel and cetyltrimethylammonium bromide (CTAB; e.g. Gachon et al. 2009) buffer for further molecular study. Representative herbarium specimens and permanent slides were deposited at the East Mediterranean Seaweed Herbarium at the Hellenic Centre for Marine Research (HCMR) in Anavyssos, Athens.

DNA extraction, PCR amplification and sequencing

DNA extractions were carried out using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) on material from specimens preserved in silica gel. From *Dictyota cyanoloma*, the partial mitochondrial cytochrome oxidase I (*COI*) was amplified using either the primer pair COI-789F & COI-1378R (Silberfeld et al. 2010) or *cox1*-GazF1 & *cox1*-GazR1 (Saunders 2005).

PCR amplification was performed in a total volume of 25 µl, containing 1.25 units µl⁻¹ (0.25 µl stock) of Taq DNA Polymerase (Promega), 5 µl GoTaq™ buffer, 5 mM MgCl₂, 1.25 mM dNTPs (desoxy nucleotides), 1.87 mM of each primer and 1 µl of template DNA (5–50 ng µl⁻¹). For *Dictyota cyanoloma*, PCR amplification was conducted with the primer pair *cox1*-GazF1/*cox1*-GazR1 (Saunders 2005).

PCR amplification was carried out with an initial denaturation at 94°C for 2 min; followed by 35 cycles of amplification consisting of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and elongation at 72°C for 1 min, followed by a final extension at 72°C for 5 min. Returned chromatograms (in ABI/Applied Biosystems format) were imported into BioEdit (Hall 1999) for quality control and resulting consensus sequences were queried against the GenBank online database using BLASTn (Altschul et al. 1997).

For *Sebdenia monnardiana*, the markers *rbcL*, 3'-COI and SSU were used. *rbcL* was amplified using the primer pair RbcL77F (Yang et al. 2014) – RbcL952R (Kawai et al. 2007, as Ral-R952), 3'-COI was amplified with primers *cox1*-789F and *cox1*-1378R (Silberfeld et al. 2010), and SSU with primers NS1F-NS4R (White et al. 1990), AFP2F (Burkhardt and Peters 1998) and AFP1R (5'-GGTAATGATCCTTC-CGCAG-3'). PCR was performed in a total volume of 25 µl, with 21.5 µl of Taq Master Mix stock (VWR International, Haasrode, Belgium), 1.0 µl of each primer (10 mM) and 1.5 µl of template DNA. For primers RbcL77F-RbcL952R, PCR included an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 30 s and elongation at 72°C for 1 min, followed by a final extension at 72°C for 5 min. For primers NS1F-NS4R and AFP2F-AFP1R, PCR included an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 30 s and elongation at 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCR success was confirmed by 1.2% agarose gel electrophoresis, and visualised by SYBR safe DNA gel stain (Invitrogen/ThermoFisher Scientific, Waltham, MA, USA) under UV light. Acquired PCR products were purified using the GeneJET PCR purification Kit (ThermoFisher Scientific, Waltham, MA, USA) and sent off to be sequenced by an external Sanger sequencing service (Source Biosciences, Nottingham, UK). Returned chromatograms were curated similarly as with *Dictyota cyanoloma*.

Molecular phylogeny

Phylogenies for *Sebdenia monnardiana* were calculated separately for SSU and *rbcL* regions. Sequences including other relevant taxa of the Sebdeniales and Rhodymeniales (Table S1 for SSU, Table S2 for *rbcL*) were aligned and annotated in BioEdit (Hall 1999), and Maximum Likelihood (ML) trees were generated using MEGA7 (Kumar et al. 2016). The ML tree was generated based on the GTR model with Gamma distributed rates among sites, with invariant sites included. One hundred bootstrap replicates were used to assess tree robustness. Members of

Table 1: Stations and dates of ROV surveys covered here, (A) March 2013 and (B) October 2013.

Dive no.	Date	Location	Surveyed depth range
A			
1	11/03/2013	N 38 07.570, E 020 27.839 Off Argostoli Gulf (SE), Kefalonia	44–104 m
2	11/03/2013	N 38 06.769, E 020 27.726 Off Argostoli Gulf (SE), Kefalonia	77–90.5 m
3	12/03/2013	N 38 27.251, E 020 36.343 Ithaki Straits (N)	95–99 m
4	13/03/2013	N 39 12.716, E 020 15.030 Off Paxoi Island (E)	60–79.5 m
5	13/03/2013	N 39 02.582, E 020 14.225 Off Antipaxoi Island (SW)	65–79 m
6	13/03/2013	N 39 27.068, E 020 12.695 Off Xeronisi islet, Corfu Straits (SE)	55.1–61 m
7	13/03/2013	N 39 27.194, E 020 12.372 Off Xeronisi islet, Corfu Straits (SE)	54–63 m
8	13/03/2013	N 39 27.138, E 020 12.307 Off Xeronisi islet, Corfu Straits (SE)	39.5–59 m
9	14/03/2013	N 39 39.022, E 019 55.533 Off Vidos islet, Corfu Straits (NW)	25–31.6 m
10	15/03/2013	N 39 53.088, E 019 36.054 Off Ereikoussa Island (E)	14–36 m
11	15/03/2013	N 39 44.907, E 019 57.629 N. Corfu Straits	23.5–38 m
12	15/03/2013	N 39 45.447, E 19 57.203 N. Corfu Straits	62–66.2 m
B			
1	20/10/2013	N 38 47.971, E 22 50.229 Lihadonissia – Strongili Straits	68–69.5 m
2	20/10/2013	N 38 47.107, E 22 49.334 Strongili Islet (South) – Knimidhas Straits	58.3–54 m
3	20/10/2013	N 38 50.256, E 23 02.055 Strongili Islet (South) – Knimidhas Straits	92–90 m; 53–51 m
4	21/10/2013	N 38 54.888, E 22 55.292 Off Agios Konstantinos	66–68 m
5	21/10/2013	N 38 56.176, E 23 00.249 Oreoi Straits (Glyfa – Agiokampos)	70.2–54 m
6	21/10/2013	N 39 00.027, E 23 05.050 Off Argyronissi Islet (South)	70 m
7	21/10/2013	N 38 59.931, E 23 04.801 Off Argyronissi Islet (South)	75.1–73 m
8	22/10/2013	N 39 02.373, E 23 20.980 Prassonisi Islet South	88–40 m
9	22/10/2013	N 39 12.714, E 23 30.073 Off N Skiathos Island	75.6–59 m
10	23/10/2013	N 39 15.081, E 23 19.984 Off East Pelio	68–44 m
11	23/10/2013	N 39 16.768, E 23 20.421 Off East Pelio (deep)	104–101 m
12	23/10/2013	N 39 16.316, E 23 20.438 Off East Pelio	96.6–92 m
13	24/10/2013	N 39 05.659, E 23 02.775 Trikeri Lighthouse	70–76 m
14	24/10/2013	N 39 05.201, E 23 07.529 Off Southern Pelio	64–17 m
15	24/10/2013	N 39 03.707, E 23 21.516 Off Southern Pelio	64–43 m; 39–76 m

Rhodymeniales were used as outgroups in the analyses, *Perbella minuta* (Kyllin) Filloramo *et* G.W. Saunders and *Hymenocladia chondricola* (Sonder) J.A. Lewis in the SSU phylogeny and *Rhodymenia pseudopalmata* (J.V. Lamouroux) P.C. Silva and *Dictyothamnion saltatum* A.J.K. Millar in the *rbcL* phylogeny.

Results

Despite intensive survey efforts totalling 27 ROV dives in the areas where the prediction model had suggested its occurrence, over 10 days during 2 separate cruises, *Laminaria rodriguezii* was not detected at any of the sites, neither in the Ionian Sea nor in the NW Aegean.

Dictyota cyanoloma, easily recognisable by the characteristic blue iridescence of its thallus margins (Figure 2), was found during snorkelling surveys in a shallow-water community at approx. 0.5–1 m depth on a rocky coast of SW Kefalonia in Argostoli Bay (Kefalonia Island – 38.202022° N; 20.4859361° E) together with *Dictyota dichotoma* var. *intricata* (C. Agardh) Greville and *Ellisolandia elongata* (J. Ellis *et* Solander) K.R. Hind *et* G.W. Saunders, adjacent to ROV station 1 (Figure 2), on March 11, 2013. A specimen was deposited in the East Mediterranean Seaweed Herbarium, Athens (110313-6 HCMR Herbarium). The taxon was easily identified given the blue iridescence of its margins in sunlit shallow waters. The COI sequence (Table 2) showed complete identity to those obtained from specimens in Spain (Tronholm *et al.* 2010).

Sebdenia monnardiana was found in a rhodolith bed at 60.3 m depth off northern Euboia (N 39 03.707, E 23 21.516) during ROV dive no. 15 on Oct. 24, 2013 (Figure 3A). It was about 12 cm wide and 10 cm tall, and attached



Figure 2: *Dictyota cyanoloma* in situ on the coast of SW Kefalonia.

through a small holdfast on a coralline boxwork rhodolith. The morphological characteristics were cartilaginous texture, reddish colouration (but turning yellowish when dried as a herbarium specimen), few anastomoses of the lobes present, and a smooth margin. In cross section (about 500 µm thick), an interwoven filamentous medulla (filaments about 10 µm in diameter) surrounded by layers of cortical cells could be observed. Inner cortical cells were bigger, about 60–70 µm in diameter, while outer cortical cells were small, about 3 µm in diameter in surface view. Double connections among the inner

Table 2: Nucleotide sequences obtained in this study and corresponding database accession numbers.

Taxon	Locus	Accession numbers
<i>Dictyota cyanoloma</i>	COI	LT618775
<i>Sebdenia monnardiana</i>	<i>rbcL</i>	MN642085
	COI	MN642084
	SSU	MN533961

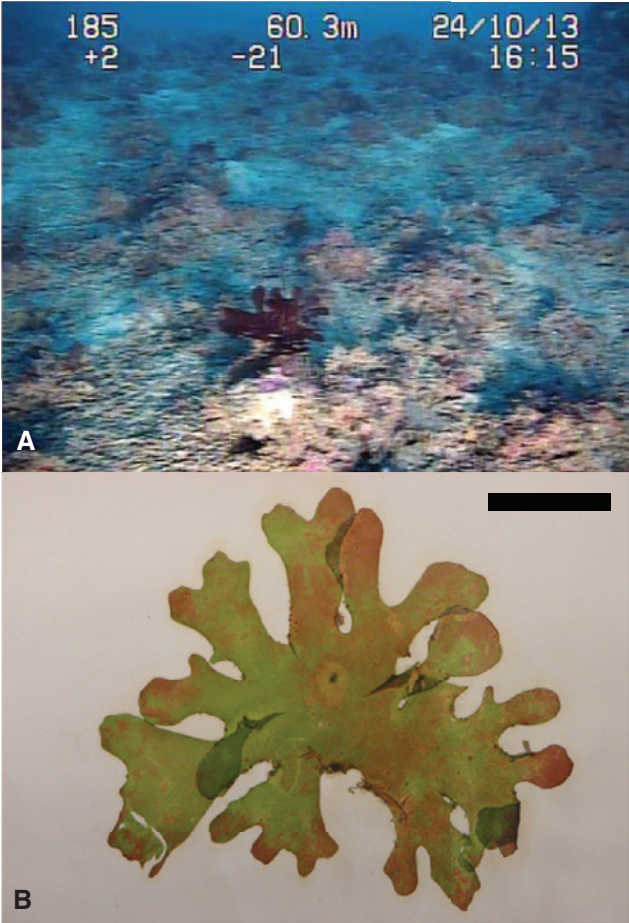


Figure 3: *Sebdenia monnardiana*. (A) In situ at 60.3 m depth off northern Euboia. (B) Herbarium specimen (scale bar: 3 cm).

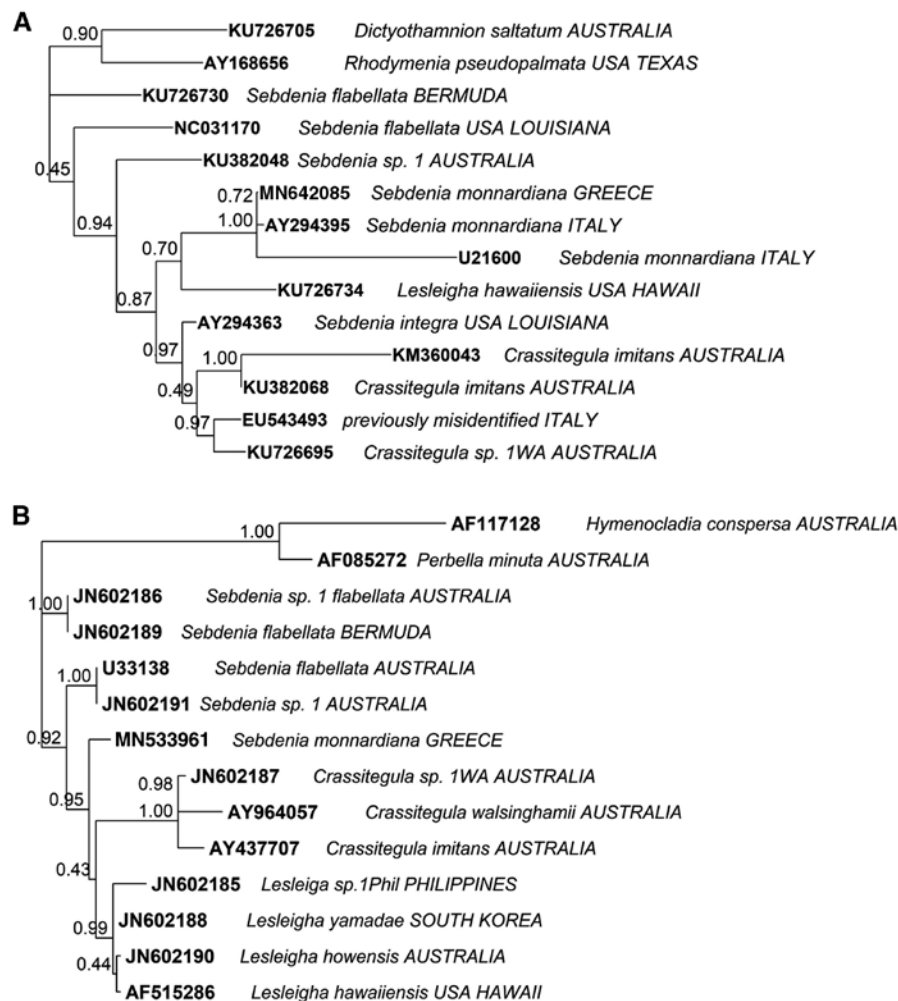


Figure 4: Maximum likelihood phylogenies of *rbcL* (A) and SSU (B) for the *Sebdenia monnardiana* specimen reported here (marked "GREECE"). Values close to nodes represent their support from 100 bootstrap replicates.

cortical cells were present. No reproductive organs were observed. A specimen was deposited in the East Mediterranean Seaweed Herbarium, Athens (241013-1 HCMR Herbarium; Figure 3B). A complete sequence of SSU as well as partial sequences of *rbcL* and COI were obtained (Table 2). Maximum Likelihood phylogenies were calculated for *rbcL* (Figure 4A) and SSU (Figure 4B).

Discussion

Deep-water kelp in Greece?

Despite the survey efforts within the framework of the two cruises covered here, the question of the existence of

Laminaria rodriguezii in east Mediterranean waters has to be considered unresolved. As of now, *L. rodriguezii* has never been observed in Greek waters. Previous records of *Laminaria digitata* (Hudson) Lamouroux and *Saccharina latissima* (Linnaeus) Lane, Mayes, Druehl & Saunders from the Ionian Sea cannot be verified due to loss of these materials during World War II. These possibly referred to *Saccorhiza polyschides* and not to deep-water kelps because they were based on specimens cast ashore together with seagrass leaves. The absence of observations during the cruises does not constitute conclusive negative evidence considering the patchy occurrence in areas such as the Adriatic where numeric prediction modelling would suggest a more contiguous occurrence (Žuljević et al. 2016). Also, the intense bottom trawling activity in part of the Greek waters has to be taken into account. Even

if *L. rodriguezii* had existed in any of the areas surveyed here, its communities may well have been exterminated in recent decades, in analogy to its disappearance from >95% of its previously-documented range in the Adriatic Sea due to bottom trawling (Žuljević et al. 2016). Indeed, at several of the sites, the impacts of bottom trawling (trawling scars) were obvious during the ROV surveys.

Another hypothesis that could potentially account for the lack of *Laminaria rodriguezii* observations during the surveys reported here is that the model (Graham et al. 2007), which was developed and successfully tested for tropical locations (e.g. Galapagos), insufficiently reflects the strongly seasonal environmental conditions of the Mediterranean, and that the model may need further refinement. Specifically, although the model developed by Graham et al. (2007) predicted locations in our study region where deep *L. rodriguezii* may occur, if *L. rodriguezii* is less tolerant of lower irradiance levels than the physiological parameters used in the model, then the predicted areas in Greek waters may have been over-estimates of where *L. rodriguezii* could persist.

Without doubt, the ROV surveys conducted within the framework of this study have to be considered insufficient for conclusively answering the question of the occurrence of *Laminaria rodriguezii* in eastern Mediterranean waters. We suggest that fishermen, ROV operators and divers operating in the depth range of 40–150 m for unrelated purposes in the eastern Mediterranean should be given adequate instructions to be able to recognise this species in case of chance encounters.

Dictyota cyanoloma

The recently described *Dictyota cyanoloma* (Dictyotales, Phaeophyceae; Tronholm et al. 2010) is known from the Mediterranean coast of Spain (Tronholm et al. 2010, Bárbara et al. 2015), the Adriatic, Portugal, Madeira, the Azores and Canary Islands (Tronholm et al. 2010). Its phylogenetic affinities have been established by sequences of partial LSU rDNA, *rbcL*, *psbA*, *cox1*, *cox3*, and *nad1* (Tronholm et al. 2010). Recent findings from Turkey (Taskin 2013) constitute the first records for the eastern Mediterranean so far.

The record of this taxon from Kefalonia Island reported here supports the notion that the eastern Mediterranean remains incompletely surveyed for its macroalgal diversity. DNA sequences unambiguously confirmed that the material belongs to *Dictyota cyanoloma*. In the eastern Mediterranean, *D. cyanoloma* has been previously reported from the Aegean coast of Turkey near Izmir (Taskin 2013), but not from Greek waters. This record suggests a contiguous,

albeit rare distribution of this taxon in the shallow infralittoral from the Azores, Madeira and the Canary Islands, and throughout the Mediterranean, including the Adriatic to the Mediterranean coast of Turkey. Recent work (Steen et al. 2017) has shown that this taxon is not native to Europe, but an introduction which has been present in the Adriatic as early as 1935. The same study (Steen et al. 2017) showed that *D. cyanoloma* is also present in western and southern Australia.

Sebdenia monnardiana

Sebdenia monnardiana is known only from circalittoral habitats of the western Mediterranean – but not yet from the eastern Mediterranean. No SSU sequences, only *rbcL* sequences are available for *S. monnardiana* so far.

The finding presented here constitutes a new record for Greece and, indeed, the entire eastern Mediterranean. The Sebdeniaceae was erected by Kylin (1932) in order to accommodate *Sebdenia monnardiana* originally described from the North African coast of the western Mediterranean. Athanasiadis (2002; p. 120) reported it to be “the largest Mediterranean red alga, reaching 1 meter in diameter”. Compared to this, the specimen reported here (of only about 10 cm size) is very small. The SSU sequence reported here is the first sequence of this important marker for *S. monnardiana*, which is significant since *S. monnardiana* is the type species for the genus *Sebdenia* and the order Sebdeniales (Withall and Saunders 2006), and it is clearly difficult to collect. Our *rbcL* sequence matches two sequences reported previously for this taxon from Italy (especially AY294395.1 with 99.53% identity for 100% query cover, but also U21600.1, with 98.94% identity). The specimen corresponding to AY294395 was collected at Lachea Island, near Catania, Italy, by G. Furnari and M. Cormaci in October 1994, and is surely a correctly identified *S. monnardiana* (Gavio et al. 2005). The lower similarity value for U21600 is due to missing data and possible errors in this older sequence; nevertheless it forms a clade with AY294395 and our sequence. Sequence similarity of our specimen with U21600 and AY294395 confirms the morphological identification of our material. In contrast, the sequence EU543493 for an alga from central Italy is problematical. It was submitted to NCBI by R. D’Archino, N. Abdelahad, and G. Procaccini and is only 89.4% identical with EU543493 and 90.6% with our sequence. However, it appears to be a good *rbcL* sequence (most substitutions are at third base positions) and likely belongs to a related species of as yet unknown identity, which in our

rbcl tree (Figure 4A) forms a clade with *Crassitegula* sp. from Australia.

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Bionotes



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